A Quantitative Study of Pinocytosis and Lysosome Function in Experimentally Induced Lysosomal Storage

By ANNE V. S. ROBERTS, SUSAN E. NICHOLLS, PENELOPE A. GRIFFITHS,
KENNETH E. WILLIAMS and JOHN B. LLOYD

Biochemistry Research Unit, University of Keele, Keele, Staffordshire ST5 5BG, U.K.

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The highly pinocytic epithelial cells of the visceral yolk sac from 17.5-day rat conceptuses were used as a model in which to induce engorgement of the vacuolar system by direct accumulation of substances that are not hydrolysed by lysosomal enzymes. The ultrastructural appearances of these cells in pregnant animals that 24–48 h before had received intraperitoneal injections of Triton WR-1339, polyvinylpyrrolidone, dextran or sucrose revealed gross abnormalities that were confined to the vacuolar system; in comparison with normal tissue the number, and in some cases the size, of vacuoles was increased, leading to close packing within the apical cytoplasm and distortion of the normal rounded shape. By culturing yolk sacs *in vitro*, rates of ingestion of ^{125}I -labelled polyvinylpyrrolidone and of ^{125}I -labelled bovine serum albumin were determined, together with the rate of digestion of the labelled protein. The rates of exocytosis of ^{125}I -labelled polyvinylpyrrolidone and of lysosomal enzymes were also determined. No significant differences between normal and highly vacuolated tissues were found. Apparently marked vacuolation of these cells by these agents is without significant effect on pinocytosis, exocytosis or intralysosomal proteolysis.

The lysosomal storage diseases (Hers & Van Hoof, 1973) comprise a heterogeneous group of congenital disorders with one common feature, the intralysosomal accumulation of biopolymers. The diseases are progressive, many being fatal in early life. They vary in their severity, in the organ(s) most affected, and in the nature of the substance accumulated. Morphological examination of affected tissues reveals cells filled with numerous large membrane-bound vacuoles. In several of the diseases the accumulation of material has been correlated with either decreased activity or complete absence of some lysosomal enzyme.

It is not known how an increasing distention of the vacuolar system leads to cell damage and ultimately to the failure of some vital organ. The membrane of engorged lysosomes might become abnormally permeable or fragile, with consequent release of lysosomal enzymes into the soluble cytoplasm. Or engorgement of the vacuolar system might impair endocytosis or intracellular fusion of vacuoles, e.g. of phagosomes and lysosomes, causing a generalized failure of lysosomal function. Or the accumulated substances might impair the performance of normal lysosomal enzymes, either by direct inhibition or indirectly by altering some feature of the intralysosomal environment, such as its pH.

Experimental storage conditions, in which the ultrastructure of tissues closely resembles that found in tissue from patients with storage disease, can be

produced by the administration of indigestible lysosomotropic agents (de Duve *et al.*, 1974). Dextran, Triton WR-1339 and sucrose (Lloyd, 1973) are well-documented examples of agents that accumulate in the vacuolar system of tissues with a high endocytic capacity. Lysosomal storage can also be produced indirectly, as in the accumulation of lipid material in vacuoles after administration of such drugs as chloroquine (Abraham *et al.*, 1968; Fedorko *et al.*, 1968) and chlorpromazine (Brosnan *et al.*, 1970).

Williams *et al.* (1975*a,b*) have described an organ culture system in which the rate of pinocytosis of radioactively labelled macromolecules may be measured with precision. The radioactively labelled substrate chosen may be either one that is resistant to or one that is susceptible to intralysosomal digestion; if the latter, it is possible to measure the rate of its intralysosomal catabolism. The tissue used is the 17.5-day rat visceral yolk sac, a membranous structure whose outer uterine luminal surface comprises a highly pinocytic columnar epithelium. The observation of Schultz *et al.* (1966) that maternal injection of Triton WR-1339 induced an engorgement of the vacuolar system in the visceral yolk-sac epithelium suggested the possibility of using the system developed *in vitro* by Williams *et al.* (1975*a,b*) to compare aspects of lysosomal function in both normal and experimentally engorged tissue.

A preliminary report of some of this work has been published (Roberts *et al.*, 1974).

Methods

Induction of experimental lysosomal storage in rat yolk sac

Four substances were used to induce an engorged vacuolar system in the visceral yolk-sac epithelial cells: Triton WR-1339 (Hilton Davis Chemicals, Newcastle upon Tyne, U.K.), polyvinylpyrrolidone (PVP-40; mean mol.wt. 40000; Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.), dextran (mean mol.wt. 500000; Sigma Chemical Co.) and sucrose (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.).

Inbred Wistar rats were mated overnight and, if a vaginal plug was observed next morning, pregnancy was timed from 24.00h. The four vacuolating agents, dissolved in small volumes of 1% (w/v) NaCl, were administered at different stages of pregnancy before 17.5 days of gestation in a variety of regimes of dose, route and time, in order to determine the combination that reproducibly gave the most profound alteration in the morphology of the vacuolar system.

Examination of yolk sacs by electron microscopy

Normal and treated rats were killed by cervical dislocation on day 17.5 of gestation. Yolk sacs were removed immediately, placed in Karnovsky's (1965) fixative at pH 7.4 and cut into small fragments. The tissue was then transferred into fresh fixative for 2h at 4°C, washed overnight in 0.2M-potassium phosphate buffer (pH 7.4) containing sucrose (0.44M), post-fixed for 2h in 2% (w/v) OsO₄ in 0.2M-potassium phosphate buffer, and finally dehydrated through acetone and embedded in Araldite CY 212. Thin (pale-gold) sections were cut by using glass knives with a Reichert OMU 2 ultramicrotome and stained with aq. uranyl acetate and lead citrate before examination in a Phillips EM 200 electron microscope at an accelerating voltage of 60kV.

Release of enzymes from yolk sacs cultured in vitro

Day-17.5 rat yolk sacs were incubated at 37°C with shaking in 15ml of culture medium comprising medium TC 199 containing 10% (v/v) calf serum under O₂+CO₂ (95:5). At intervals of 0.25, 0.5, 1, 2, 4 and 6h, samples (0.75ml) of the medium were removed and the flasks re-gassed. The medium samples were centrifuged at 1500g for 15min to remove cell debris and stored at -20°C before assay for β -galactosidase, β -N-acetylglucosaminidase and lactate dehydrogenase. The enzyme activities of each sample, after correction for the endogenous activity of the culture medium, were used to calculate the total amount of enzyme released into the medium up to the time of sampling. At the end of the incubation the yolk sacs were homogenized at 4°C for 30s in 5ml of 0.01M-potassium phosphate buffer, pH 7.4, by using a Virtis homogenizer (Virtis Research Equipment,

Gardiner, NY, U.S.A.) set at speed 2. The homogenates were assayed immediately for β -galactosidase, β -N-acetylglucosaminidase and lactate dehydrogenase; by adding to these values the total enzyme released into the medium by the end of the incubation an estimate of the total enzyme initially present in the yolk sac before culture was obtained.

To test the stability of the three enzyme activities to the incubation conditions, a normal 17.5-day yolk sac was incubated as above for 4h. The yolk sac was removed and the resulting culture medium centrifuged at 1500g for 15min. The supernatant medium was re-gassed and incubated for a further 6h. At intervals of 0.5, 2, 4 and 6h samples (0.75ml) of the medium were removed and assayed for β -galactosidase, β -N-acetylglucosaminidase and lactate dehydrogenase activities.

β -N-Acetylglucosaminidase was assayed by using 2.5mM-4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside (Koch-Light Ltd., Colnbrook, Bucks., U.K.) as substrate in 0.1ml of citrate/phosphate buffer, pH 4.3 (0.1M-sodium citrate adjusted to the appropriate pH with 0.2M-sodium phosphate). To this was added culture medium (10–50 μ l) and the mixture was incubated at 37°C for 15min. The reaction was terminated by the addition of 2.8ml of 0.17M-glycine carbonate buffer, pH 10.5. The fluorescence was determined in a Perkin-Elmer 1000 fluorimeter by using an excitation wavelength of 365nm and an emission wavelength of 450nm. Standards containing up to 1nM-4-methylumbelliferone in glycine carbonate buffer, pH 10.5, were included with each assay. β -Galactosidase was assayed similarly by using 2.5mM-4-methylumbelliferyl β -D-galactopyranoside as substrate in 0.1ml of citrate/phosphate buffer, pH 3.2. Lactate dehydrogenase was assayed by the method of Lowry *et al.* (1957).

Release of ¹²⁵I-labelled polyvinylpyrrolidone from yolk sacs cultured in vitro

To determine the extent of exocytic regurgitation by yolk sacs from rats that had received vacuolating agents, 17.5-day yolk sacs were incubated for 4h in the presence of ¹²⁵I-labelled polyvinylpyrrolidone (25 μ g/ml), washed in fresh medium to remove extracellular radioactivity, and then re-incubated for 4h in substrate-free medium. Full details of the method are given by Williams *et al.* (1975a).

Uptake and digestion of macromolecules by rat yolk sacs cultured in vitro

The method of Williams *et al.* (1975a,b), in which 17.5-day rat yolk sacs are cultured in the presence of ¹²⁵I-labelled polyvinylpyrrolidone or ¹²⁵I-labelled bovine serum albumin, was used, with minor modification. Substrate concentrations were 5.0 μ g/ml for ¹²⁵I-labelled polyvinylpyrrolidone and 1.0 μ g/ml for

EXPLANATION OF PLATE I

Apical region of 17.5-day yolk sacs from normal rats and rats treated with vacuolating agents

Untreated rat (a). Rats that had received an intraperitoneal injection of Triton WR-1339, (500 mg/kg) 48 h before death (b), polyvinylpyrrolidone (1 g/kg) 36 h before death (c), dextran (1 g/kg) 24 h before death (d), or sucrose (1 g/kg) 24 h before death (e). Bar lines represent 1 μ m.

¹²⁵I-labelled bovine serum albumin. The total radioactivity and the protein content of each yolk sac were measured after solution in 5 ml of 1 M-NaOH for 1 h at 37°C, a more reliable method than homogenization (Williams *et al.*, 1975a) when the resolution of trichloroacetic acid-soluble and trichloroacetic acid-insoluble radioactivity in the tissue is not needed.

The rates of uptake of radioactively labelled substrates by yolk sacs cultured *in vitro* were expressed as Endocytic Indices, defined (Williams *et al.*, 1975a,b) as the volume (μ l) of medium whose contained substrate has been captured per hour by a unit quantity of yolk-sac tissue. This form of expression has the merit of normalizing results both for variation in size of yolk sacs and for the effects of decay of the radioactive label.

Results

Ultrastructural changes in yolk sacs of treated rats

Membrane-bounded vacuoles filled with homogeneous material of various moderate electron densities were abundant in the apical cytoplasm of the epithelial cells of yolk sac from normal animals. Vacuole profiles were rounded, with diameters in the range 0.8–1.8 μ m, and were separated by cytoplasmic ground substance containing other organelles (Plate 1a). These observations are concordant with those of Padykula *et al.* (1966).

Maternal administration of Triton WR-1339, polyvinylpyrrolidone, dextran or sucrose resulted in changes in the apical vacuolar system without significant alteration in the appearance of the rest of the cell. In all cases vacuoles occupied a greater proportion of the apical cytoplasm, but their detailed appearance depended on the substance administered and the extent of the changes on the dose, route and time of its administration.

Administration of either Triton WR-1339 or polyvinylpyrrolidone resulted in the appearance of vacuoles whose contents were electron-lucent but which occasionally included regions of moderate electron density resembling the vacuolar contents of normal cells (Plates 1b and 1c). The vacuolar profiles were larger than normal (diameter in range 1.0–3.0 μ m), more numerous and more closely packed together, so that their shape was often distorted. Maximum effects compatible with survival of the tissue on culturing were found after intraperitoneal administration of Triton WR-1339 at 500 mg/kg body wt., 48 h before death, or of polyvinylpyrrolidone at 1 g/kg, 36 h before death.

Administration of dextran increased the number of vacuoles in the apical cytoplasm, so that they appeared tightly packed, but caused little if any increase in their size. The vacuoles contained material that appeared as granules of moderate electron density embedded in an electron-lucent matrix, and re-

sembled glycogen in its ultrastructural appearance. Homogeneous material similar to that found in normal cells remained associated with some of the vacuoles (Plate 1d). As with Triton WR-1339 and polyvinylpyrrolidone, the vacuolar contents were frequently heterogeneous, suggesting an incomplete mixing of the exogenous macromolecule and the normal contents. Maximum effects with dextran followed the intraperitoneal administration of 1 g/kg body wt., 24 h before death. The apical vacuoles from dextran-loaded cells were often surrounded by material resembling that contained in the vacuoles. It was not possible to decide whether this material was a localized accumulation of glycogen, or dextran that had leaked out of the vacuoles into the surrounding cytoplasm.

Sucrose administration also resulted in an increase in the number of vacuoles present in each cell without significantly altering the size of individual vacuoles (Plate 1e). The vacuoles were often closely packed together and as a result were very distorted in shape. The contents were homogeneous, suggesting a complete mixing of endogenous and exogenous material. The electron density of the contents varied considerably between different vacuoles, possibly reflecting the amount of sucrose present. Maximum effect was achieved with 1 g/kg body wt., injected intraperitoneally 24 h before death.

The dose regimes described above yielded remarkably uniform and reproducible results and were adopted for all subsequent experiments. The majority of cells from each yolk sac examined were equally vacuolated, and no variation in the degree of vacuolation of cells from different regions of the yolk sac was detected.

In addition to the initial morphological investigation, one yolk sac from every rat injected to provide material for culture experiments was prepared for electron-microscopical examination.

Release of enzymes

The culture medium contained low activities of β -galactosidase and β -N-acetylglucosaminidase, which remained unaltered on incubation at 37°C for 6 h. Tables 1 and 2 show the release of β -galactosidase and β -N-acetylglucosaminidase, two lysosomal enzymes, into the incubation medium over a 6 h period from control yolk sacs and from yolk sacs from animals treated with vacuolating agents. Release was progressive over the 6 h incubation periods and there were no discernible differences in results from tissue from normal and treated rats. In contrast the lactate dehydrogenase activity of the culture medium decreased when incubated in the presence of a yolk sac. Fig. 1 shows the time-course of this decrease, with a normal yolk sac, and also illustrates that the decrease is not seen if culture medium is incubated without a yolk sac. Yolk sacs from normal rats and

Table 1. Release of β -galactosidase by cultured yolk sacs from normal rats and rats pretreated with vacuolating agents

Vacuolating agents were administered as described in the caption to Plate 1. Each line of the Table shows the results for one incubated yolk sac. Enzyme released into the culture medium up to the time of sampling is expressed as a percentage of the total initial enzyme content of the yolk sac (see the Methods section).

Time (h) ... Vacuolating agent	Enzyme (% of total initial content)					
	0.25	0.5	1.0	2.0	4.0	6.0
None	2.8	3.5	4.4	5.3	8.0	8.5
	1.5	1.6	2.6	2.7	5.0	5.9
	2.9	3.5	4.0	6.0	7.6	7.5
Triton WR-1339	2.7	3.2	3.4	5.3	6.3	7.4
	1.2	1.8	2.3	2.8	4.1	5.3
	1.8	1.7	2.3	3.6	5.2	6.2
Sucrose	2.1	2.8	3.2	4.3	5.9	6.9
	3.2	3.4	5.0	5.3	8.2	8.5
	2.6	3.5	4.5	6.7	8.1	8.2
Polyvinylpyrrolidone	1.3	2.6	3.4	3.7	4.7	5.0
	2.4	2.5	2.9	3.9	5.5	9.7
	1.4	1.9	2.5	3.2	4.8	5.4
Dextran	0.6	1.2	2.0	2.9	3.9	5.0
	0.9	1.7	3.4	4.6	5.3	7.0
	2.2	2.4	3.3	4.8	7.5	7.4

Table 2. Release of β -N-acetylglucosaminidase by cultured yolk sacs from normal rats and rats pretreated with vacuolating agents

Vacuolating agents were administered as described in the caption to Plate 1. Each line of the Table shows the results for one incubated yolk sac. Enzyme released into the culture medium up to the time of sampling is expressed as a percentage of the total initial enzyme content of the yolk sac (see the Methods section).

Time (h) ... Vacuolating agent	Enzyme (% of initial content)					
	0.25	0.5	1.0	2.0	4.0	6.0
None	5.9	6.5	7.2	8.9	10.9	10.4
	0.9	2.7	4.1	5.2	8.1	11.5
	2.5	3.0	5.1	7.4	10.1	11.5
Triton WR-1339	2.5	4.9	5.8	6.4	9.5	10.0
	2.7	5.4	7.2	10.6	10.9	11.5
	2.8	2.9	4.0	4.9	9.3	10.0
Sucrose	2.2	3.6	4.2	7.0	7.8	7.9
	4.5	4.6	6.1	7.8	8.8	9.6
	3.2	3.5	4.1	6.3	7.7	9.0
Polyvinylpyrrolidone	1.0	2.9	3.6	3.9	6.8	8.4
	0.3	1.1	3.4	4.8	4.9	8.1
	0.2	0.8	3.6	4.0	5.9	6.5
Dextran	0.4	0.9	3.2	3.6	3.9	5.9
	1.1	3.0	3.4	6.5	8.3	9.9
	2.7	2.8	3.3	5.2	5.7	7.3

rats treated *in vivo* with vacuolating agents yielded similar results in this experiment. The disappearance of lactate dehydrogenase from the culture medium on incubation with a yolk sac could reflect adsorption or pinocytotic ingestion of the enzyme by the tissue. The time-course of the phenomenon favours the first explanation.

Fig. 2 shows the extent to which enzyme activity was lost when culture media in which a yolk sac had been incubated for 4 h was re-incubated alone for a further 6 h. Lactate dehydrogenase, which was serum- and not yolk-sac-derived, lost activity at approx. 1% per h. The lysosomal enzymes, of which over 90% were derived from release from the yolk sac

Fig. 1. Loss of serum-derived lactate dehydrogenase activity from tissue-culture medium on incubation with a yolk sac

○, Incubation of medium without a yolk sac; ●, incubation with a yolk sac.

Fig. 2. Loss of enzymic activities from tissue-culture medium in which a yolk sac had been incubated, on removal of the yolk sac and further incubation

Initial incubation in the presence of a yolk sac was for 4 h. ○, Lactate dehydrogenase; △, β-galactosidase; ●, β-N-acetylglucosaminidase.

during the preincubation, lost activity at a rate of approx. 2% per h with β-galactosidase and 3% per h with β-N-acetylglucosaminidase. These values imply that the values in Tables 1 and 2 for enzyme released by cultured yolk sacs into their culture medium will

tend to underestimate slightly the actual release. However, this effect will be counterbalanced by a probable underestimate of the total initial enzyme content of the yolk sac, arising because some tissue enzyme must be present in the small pellet of detached cells always seen when culture media were centrifuged.

Exocytosis of ^{125}I -labelled polyvinylpyrrolidone

Fig. 3 shows the release of radioactivity into the medium from three normal yolk sacs containing ^{125}I -labelled polyvinylpyrrolidone that had been accumulated *in vitro*. As found by Williams *et al.* (1975a), the release of radioactivity fell into two well-defined phases. An initial rapid release of radioactivity within 1 h of re-incubation (most within 30 min) was followed by a slower but linear release over the remaining 3 h. As indicated by Williams *et al.* (1975a), the first phase of release probably reflects the washing-off of occluded or adsorbed radioactivity remaining after the standard washing procedure and the second phase results from the combined effects of exocytosis and cell death. Essentially similar results to those of Fig. 3 were obtained by using yolk sacs from animals

Fig. 3. Release of radioactivity into the medium on culturing yolk sacs that had been preincubated in the presence of ^{125}I -labelled polyvinylpyrrolidone

Preincubation with ^{125}I -labelled polyvinylpyrrolidone was for 4 h. Radioactivity released into the culture medium is expressed as a percentage of the radioactivity associated with the tissue at the time of its transfer to fresh medium containing no ^{125}I -labelled polyvinylpyrrolidone. The rate of release beyond 1 h is calculated by linear regression analysis of all values from samples taken in the 1–4 h period (i.e. excluding points from the first 60 min); the intercept on the ordinate, obtained by extrapolation of this line gives an estimate of the radioactivity released in the initial rapid phase of release. Three separate experiments are illustrated.

that had been treated with one of the vacuolating agents (Table 3); there was more variation in the extent of the initial release of radioactivity, as indicated by the intercept on the ordinate axis, than in the rate of release during the second phase.

Since the rate of exocytosis of ^{125}I -labelled polyvinylpyrrolidone in treated tissue does not differ from that in normal tissue, and since in no case is the rate of loss greater than 3% per h, the rate of accumulation of ^{125}I -labelled polyvinylpyrrolidone by yolk sacs in the experiments described below can be equated with its rate of ingestion (see also Williams *et al.*, 1975a).

Fig. 4 shows the accumulation of ^{125}I -labelled polyvinylpyrrolidone, over a 6.5 h incubation period, by yolk sacs taken from a normal rat. Each point corresponds to data relating to a single yolk sac and the straight line was fitted by a linear-regression analysis. In a total of nine similar control experiments, in each of which 6–12 yolk sacs from the same animal

were used, the rate of uptake was substantially constant and gave a mean Endocytic Index of 1.46 ± 0.21 (s.d.) $\mu\text{l/h}$ per mg of yolk-sac protein (Table 4). The correlation coefficients in such plots were in all cases in excess of 0.96.

Results from similar experiments but using yolk sacs from rats that had received vacuolating agents are also shown in Table 4. In each case the plot of uptake of substrate against time appeared linear over a 6.5 h period, although the associated correlation coefficients tended to be slightly lower. The mean values of Endocytic Index were not significantly different from those of controls.

Uptake of ^{125}I -labelled bovine serum albumin

Uptake of ^{125}I -labelled bovine serum albumin *in vitro* by rat yolk sac is followed by intracellular degradation of the protein and release of breakdown products into the medium (Williams *et al.*, 1975b).

Table 3. Release of ^{125}I -labelled polyvinylpyrrolidone by cultured yolk sacs from normal rats and rats pretreated with vacuolating agents

Vacuolating agents were administered as described in the caption to Plate 1. ^{125}I -labelled polyvinylpyrrolidone had been accumulated *in vitro* by the yolk sacs during a 4 h preincubation. Radioactivity released into the culture medium is expressed as a percentage of the radioactivity associated with the tissue at the time of its transfer to fresh medium. Each line of the Table shows the results for one incubated yolk sac.

Vacuolating agent	Radioactivity released per h, over 1–4 h period Mean $\pm$ s.d.	Correlation coefficient	Intercept on ordinate axis (see Fig. 3) Mean $\pm$ s.d.
None	2.3	0.99	13.4
	1.9	0.997	13.1
	2.4	0.99	12.6
	0.7	0.99	3.1
	0.6	0.999	3.6
	0.7	0.998	3.1
} 1.43 $\pm$ 0.86			
Triton WR-1339	2.24	0.99	11.8
	2.1	0.98	18.3
	2.2	0.98	10.1
	1.5	0.98	10.7
	2.2	0.95	20.4
	3.2	0.99	12.2
} 2.24 $\pm$ 0.55			
Sucrose	1.1	0.98	7.7
	0.9	0.96	8.5
	1.02	0.99	11.6
	0.7	0.93	10.4
	1.2	0.95	15.3
	2.9	0.97	16.7
} 1.30 $\pm$ 0.80			
Polyvinylpyrrolidone	2.5	0.94	22.8
	2.5	0.98	14.6
	3.0	0.93	21.1
	0.98	0.99	10.9
	1.6	0.99	11.1
	1.1	0.95	9.3
} 1.95 $\pm$ 0.84			
Dextran	1.7	0.99	9.6
	1.7	0.95	14.2
	1.3	0.98	13.1
	2.2	0.99	8.2
	1.4	0.99	7.4
	1.1	0.94	9.1
} 1.57 $\pm$ 0.39			
} 8.20 $\pm$ 5.36			
} 13.92 $\pm$ 4.38			
} 11.70 $\pm$ 3.63			
} 13.08 $\pm$ 7.79			
} 19.27 $\pm$ 2.75

Fig. 4. Accumulation of radioactivity by yolk sacs from an untreated rat, on culture in the presence of ¹²⁵I-labelled polyvinylpyrrolidone

The ordinate axis shows the volume of culture medium whose content of radioactivity has been captured by the time indicated, normalized for the protein content of the yolk sac.

Table 4. Uptake of ¹²⁵I-labelled polyvinylpyrrolidone in vitro by yolk sacs from normal rats and rats pretreated with vacuolating agents

Vacuolating agents were administered as described in the caption to Plate 1. Each line of the Table shows the results from eight to ten yolk sacs taken from a single animal.

Vacuolating agent	Endocytic Index (μl/h per mg of protein) Mean ± S.D.	Correlation coefficient
None	1.71	0.99
	1.24	0.96
	1.82	0.99
	1.43	0.97
	1.18	0.99
	1.33	0.99
	1.45	0.99
	1.40	0.97
	1.60	0.98
Triton WR-1339	1.52	0.98
	1.74	0.99
	1.23	0.97
Sucrose	1.54	0.98
	1.19	0.96
	1.70	0.99
Polyvinylpyrrolidone	1.40	0.99
	1.87	0.94
	1.51	0.97
Dextran	1.54	0.94
	1.43	0.98
	1.65	0.94

The amount of radioactivity found in the yolk sac itself (Fig. 5, curve A) increases during the first 1–2 h of incubation and then becomes approximately constant; similarly the rate of appearance of acid-soluble radioactivity in the culture medium (Fig. 5, curve B) rises and then becomes constant. As shown by Williams *et al.* (1975b), curve C (sum of curves A and B) indicates the total amount of ¹²⁵I-labelled bovine serum albumin that has been ingested by the tissue. Since this value increases linearly with time, the Endocytic Index, which is equal to the gradient of this plot, is constant over at least the initial 6 h period. The Endocytic Index of a batch of ¹²⁵I-labelled bovine serum albumin has been shown to depend on the extent of denaturation (Moore *et al.*, 1974), and the three batches of albumin used in these experiments had different Endocytic Indices (Table 5).

By using tissues engorged with one of the four agents, the patterns of accumulation of radioactivity

Fig. 5. Accumulation of radioactivity by the tissue and appearance of trichloroacetic acid-soluble radioactivity in the culture medium, on culturing yolk sacs from a normal rat in the presence of ¹²⁵I-labelled bovine serum albumin

The ordinate axis shows the volume of culture medium whose content of trichloroacetic acid-insoluble radioactivity is found associated with the tissue in either trichloroacetic acid-insoluble or -soluble form (curve A) or is found in the culture medium as trichloroacetic acid-soluble radioactivity (curve B). Curve C is obtained by summation of curves A and B.

Table 5. Uptake of ^{125}I -labelled bovine serum albumin *in vitro* by yolk sacs from normal rats and rats pretreated with vacuolating agents

Vacuolating agents were administered as described in the caption to Plate 1. Each line of the Table shows the results from eight–ten yolk sacs taken from a single animal. Values for tissue radioactivity are means $\pm$ S.D. of post-2 h values.

Vacuolating agent	Batch of ^{125}I -labelled bovine serum albumin	Endocytic Index ($\mu\text{l/h}$ per mg of protein) Mean $\pm$ S.D.	Correlation coefficient	Tissue radioactivity ($\mu\text{l/mg}$ of protein) Mean $\pm$ S.D.
None	I	16.8	0.94	25.2 $\pm$ 3.4
		15.0	0.99	14.5 $\pm$ 4.7
		14.5	0.99	12.0 $\pm$ 2.7
None	II	19.9	0.97	17.7 $\pm$ 2.6
		21.0	0.96	20.5 $\pm$ 3.2
		34.2	0.97	17.0 $\pm$ 2.4
		27.0	0.98	21.3 $\pm$ 2.4
None	III	55.2	0.98	85.5 $\pm$ 10.7
		66.1	0.98	73.6 $\pm$ 14.6
		68.0	0.94	81.6 $\pm$ 28.4
Triton WR-1339	I	14.5	0.97	15.4 $\pm$ 2.0
		15.4	0.98	10.9 $\pm$ 1.3
		11.3	0.99	11.3 $\pm$ 1.7
Sucrose	II	19.7	0.88	21.8 $\pm$ 4.1
		24.6	0.95	22.6 $\pm$ 7.1
		24.4	0.96	22.3 $\pm$ 3.2
Polyvinylpyrrolidone	II	21.8	0.97	20.1 $\pm$ 3.2
		34.2	0.98	24.7 $\pm$ 8.1
		29.6	0.99	24.2 $\pm$ 3.8
Dextran	III	72.2	0.99	97.3 $\pm$ 18.6
		57.4	0.98	72.0 $\pm$ 11.6
		57.3	0.98	78.0 $\pm$ 9.3

within the tissue and of release of digestion products into the medium were as in the control experiments. Moreover, comparison of both the mean Endocytic Index of ^{125}I -labelled bovine serum albumin and the mean amount of radioactivity within the tissue revealed no differences from controls (Table 5). The correlation coefficients for the data in the ^{125}I -labelled bovine serum albumin experiments when using normal tissue were more variable than for similar experiments using ^{125}I -labelled polyvinylpyrrolidone as substrate, but with vacuolated tissues the correlation coefficients for ^{125}I -labelled bovine serum albumin were neither lower nor more variable than those in the corresponding control experiments.

Discussion

Williams *et al.* (1975*a,b*) have described the kinetics of pinocytic uptake of ^{125}I -labelled polyvinylpyrrolidone and bovine serum albumin by rat yolk sac in culture. The present work extends the previous findings by showing that lysosomal enzyme release by cultured yolk sacs amounts to less than 2% of the activity originally present in the tissue per h. This value is comparable with the rate of release of pinocytized ^{125}I -labelled polyvinylpyrrolidone (Williams

et al., 1975; and the present paper). Release of lysosomal enzymes and of ^{125}I -labelled polyvinylpyrrolidone could reflect either exocytosis or cell death, and it was hoped to distinguish between these alternatives by measuring the release of lactate dehydrogenase, an enzyme of the cytosol. Unfortunately the calf serum present in the culture medium contained a high activity of lactate dehydrogenase, and incubation with a yolk sac led to a decrease rather than an increase in the concentration of the enzyme in the medium. Nevertheless there is no evidence for release of lactate dehydrogenase into the medium in the period following the phase of rapid depletion of the serum-derived activity; hence the release of lysosomal enzyme and ^{125}I -labelled polyvinylpyrrolidone is likely to reflect exocytosis chiefly.

In the study reported here we have been able, by maternal administration of Triton WR-1339, polyvinylpyrrolidone, dextran or sucrose, to mimic experimentally in rat yolk-sac epithelium the major ultrastructural feature seen in tissues from human lysosomal storage disease. In organ culture such tissue showed no decrease in the rate of pinocytosis of either ^{125}I -labelled polyvinylpyrrolidone or bovine serum albumin. When using the (digestible) protein as substrate the amount of radioactivity in the vacuo-

lated tissue did not continue to rise after the first 1–2h, and was within experimental error identical with that in control tissues; this indicates that the proteolytic capacity of the tissue is not affected to any demonstrable degree by any of the treatments. Neither could any effect on the exocytosis of lysosomal enzymes or of previously pinocytized ^{125}I -labelled polyvinylpyrrolidone be demonstrated. Thus it appears that neither pinocytosis, proteolysis nor exocytosis are affected by the vacuolating regimes used.

These findings contrast with several reports where the accumulation of indigestible material in the vacuolar system has been observed to affect certain aspects of cell activity. Wagner *et al.* (1971) observed that the uptake of sucrose by Chang liver cells ceased abruptly after 3h in culture, by which time there is intense cytoplasmic vacuolation. Dingle *et al.* (1969) reported the release of large amounts of lysosomal enzymes from chick limb-bone rudiments cultured in the presence of 0.08M-sucrose. Kint *et al.* (1973) demonstrated that the decreased activity of β -galactosidase seen in certain mucopolysaccharidoses is caused by inhibition of the enzyme by the accumulated glycosaminoglycans. Just *et al.* (1974) showed that several polyanions could strongly inhibit the activity of lysosomal enzymes *in vitro*, but that the response of individual enzymes to a given concentration of a particular polyanion varied greatly. Wibo & Poole (1974) have suggested that chloroquine accumulating in its cationic form within lysosomes causes a decrease of intralysosomal proteolysis by inhibiting cathepsin B₁. Trypan Blue, suramin and aurothiomalate (Davies *et al.*, 1969, 1971; Buys *et al.*, 1973; Bertini *et al.*, 1973) inhibit intralysosomal proteolysis, although in the yolk-sac culture system Trypan Blue (Williams *et al.*, 1973, 1976) and suramin (Y. Saito, K. E. Williams & J. B. Lloyd, unpublished work) exert an inhibiting effect on pinocytosis that masks any effect on proteolysis. A recent report (Hart & Young, 1975) indicates that suramin may inhibit the fusion of phagosomes with lysosomes in mouse peritoneal macrophages.

Our observations of normal proteolysis in vacuolated tissue may be a consequence of the agents used being uncharged, and hence relatively inert compared with the charged polymers used by Just *et al.* (1974). Clearly the above study needs extending to include a number of charged vacuolating agents but so far this has proved impossible owing to the high toxicity of such agents. It would also be valuable to study catabolism by other lysosomal enzymes, such as the polysaccharide-catabolizing enzymes, but this awaits the development of suitable techniques.

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